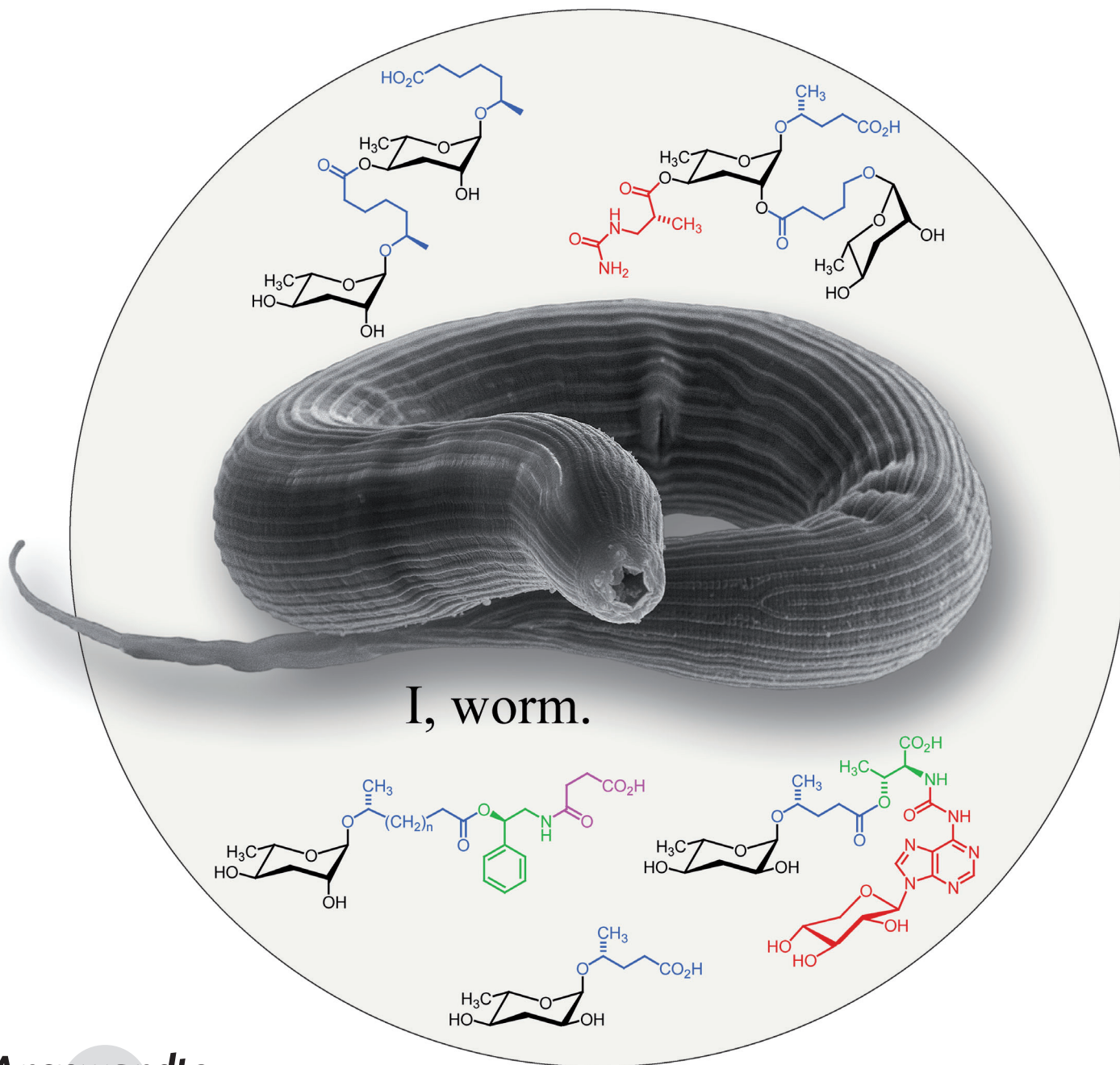


Complex Small-Molecule Architectures Regulate Phenotypic Plasticity in a Nematode**

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Small molecules serve a wide variety of essential biological functions.^[1] They facilitate inter- and intra-species chemical communication, are used for chemical defense and predation, function as hormones and second messengers in animals and plants, and serve as building blocks for biological macromolecules. In contrast to many groups of microorganisms and plants, whose genomes encode a great variety of “secondary” small-molecule biosynthetic pathways (for example, for polyketides and non-ribosomal peptides),^[2] most animals are not known to have dedicated biosynthetic pathways to generate structurally complex small molecules. Correspondingly, many unusual metabolites that have been isolated from basal animals (e.g., sponges and bryozoans, among others) and arthropods have turned out to be of microbial origin or acquired through their diet.^[3] Recent studies of the model organism *Caenorhabditis elegans* show that this nematode produces a family of small-molecule signals, the ascarosides, for example, ascr#1–3 and hbas#3 (Figure 1 a), which control multiple aspects of the life history of *C. elegans*, including larval development, mating, and social behaviors.^[4] These signaling molecules are derived from combination of the dideoxysugar ascrylose with a variety of lipid- and amino acid-metabolism-derived moieties,^[4e] suggesting that nematodes, and perhaps other animals, harbor yet unrecognized biosynthetic capabilities.

As part of a broad 2D NMR-spectroscopic screen of nematode metabolomes, we analyzed the exometabolome (the entirety of all secreted and excreted metabolites) of the necromenic roundworm *Pristionchus pacificus*. Like *C. elegans*, *P. pacificus* is a free-living nematode that is used as a model organism for the study of developmental and evolutionary biology.^[5] *P. pacificus* forms a necromenic association with beetles, which may represent a pre-adaptation to the evolution of true parasitism.^[6] *P. pacificus* exhibits two types of phenotypic plasticity that are key to its survival in the wild. Like in many other nematode species, harsh environmental conditions such as food shortage trigger developmental arrest as dauer worms (a highly stress-resistant alternate

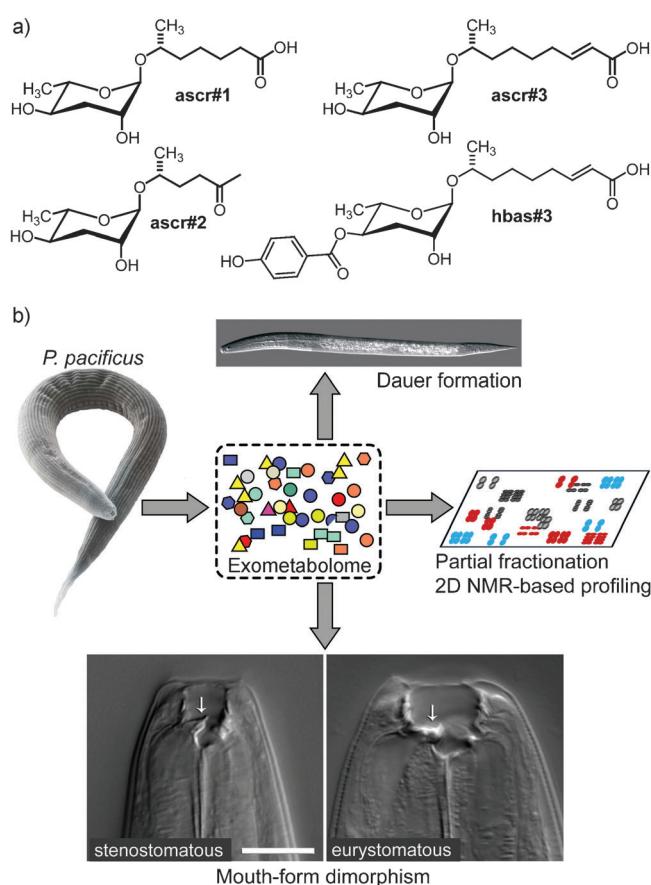


Figure 1. a) Chemical formulas of previously identified small molecules that regulate development and behavior in *C. elegans*. b) *P. pacificus* exometabolome samples induce dauer arrest and affect mouth-form dimorphism, promoting eurystomatous mouth development. Activity-guided fractionation and 2D NMR-spectroscopic profiling were used to identify the active components.

larval stage).^[7] *P. pacificus* further exhibits a unique dimorphism in mouth development, an example of phenotypic plasticity of morphology in an adult metazoan (Figure 1 b). Adult worms can have either a narrow (stenostomatous) or a wide and more complex (eurystomatous) mouth opening, the latter developing in response to conditions of low food availability. The two different mouth forms are associated with different feeding habits: stenostomatous worms are considered to feed primarily on bacteria, whereas the eurystomatous form is suited for predatory behavior toward other nematodes. Previous studies of mixed-stage *P. pacificus* cultures showed that both dauer formation and mouth dimorphism are regulated by population density,^[8] suggesting that these two phenotypes are driven by inter-organismal small-molecule signaling as part of a population density monitoring system.^[9]

2D NMR-based metabolomics of the *P. pacificus* exometabolome fractions with dauer-inducing activity (Supporting Information, Section 1.4) revealed a striking diversity of signals suggestive of primary metabolites, for example, xylose, threonine, adenosine, short-chain fatty acids, succinate, phenylethanolamine, and several dideoxysugar derivatives (Supporting Information, Figure S1); however, the NMR data

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indicated that most of these moieties are part of larger assemblies. The dideoxysugar derivatives fell into two chemically different subgroups. One group was based on ascarylose, a sugar that is widely produced by nematodes,^[10] whereas a second group of compounds appeared to include a related sugar, paratose, which previously had been reported only in bacteria (Figure S1).^[11] Analyzing the *P. pacificus* exometabolome by HPLC-MS/MS for the presence of any of the approximately 150 ascarosides recently identified from *C. elegans*,^[4e] we detected ascr#1, ascr#9, and ascr#12, as previously reported (Figure 2a).^[10] However, these three compounds accounted only for a small portion of the structures detected by 2D NMR spectroscopy.

Combining the results from NMR and high resolution HPLC-MS/MS analyses (Figure 2a, Figure S2, Table S1), we proposed structures for the major unknown components of the *P. pacificus* exometabolome, in some cases after additional fractionation to increase the content of minor components (Figure 2b). The most abundant ascaroside derivative, named pasc#9 (see Supporting Information, Section 1.1 and Ref. [23]) was proposed as an *N*-succinyl-1-phenylethanolamide linked to ascarylose by way of a 4-hydroxypentanoic acid chain (Figure 2b). This compound was accompanied by two dimeric ascaroside derivatives, a dimer (dasc#1) of the known ascr#1 in which one ascr#1 unit is attached to carbon 4 of the other ascr#1 unit, and a second dimer (ubas#1) consisting of ascr#9 to which the (ω)-oxygenated ascaroside ascr#9 is attached at position 2 (Figure 2b). This second

dimer, ubas#1, also has a 3-ureido isobutyrate moiety attached to carbon 4. To our knowledge, neither dimeric ascarosides nor ureido isobutyrate-substituted metabolites have previously been reported from nature.

These ascarosides were accompanied by two abundant metabolites that included paratose instead of ascarylose, npar#1 and part#9 (Figure 2b). In npar#1, the paratose moiety was linked to a short lipid side chain, which in turn was connected to threonine. This amino acid was connected further by way of a carbamoyl group to a derivative of the nucleoside adenosine (Figure 2b). Strikingly, this adenosine was found to include a xylopyranose, and not ribofuranose or deoxyribofuranose as in DNA, RNA, and known nucleoside-based signaling molecules. The accompanying part#9 represents the paratose and side-chain portions of npar#1 (Figure 2b).

To confirm these structural assignments, determine the stereochemistry, and explore their biological functions we developed total syntheses for each of the proposed structures, taking advantage of their modular nature (Figure 2c, Supporting Information). Comparison of the NMR spectra of the synthetic and natural samples established the configuration of the *N*-succinyl-1-phenylethanolamide moiety in natural pasc#9 as *R* (Figure S3). Similarly, comparison of the NMR spectra and HPLC retention times allowed us to assign the stereochemistry of the dimeric ascarosides, dasc#1 and ubas#1 (Figure S4, S5). The *R*-configuration of the 3-ureido isobutyrate moiety in ubas#1 is consistent with its likely origin from

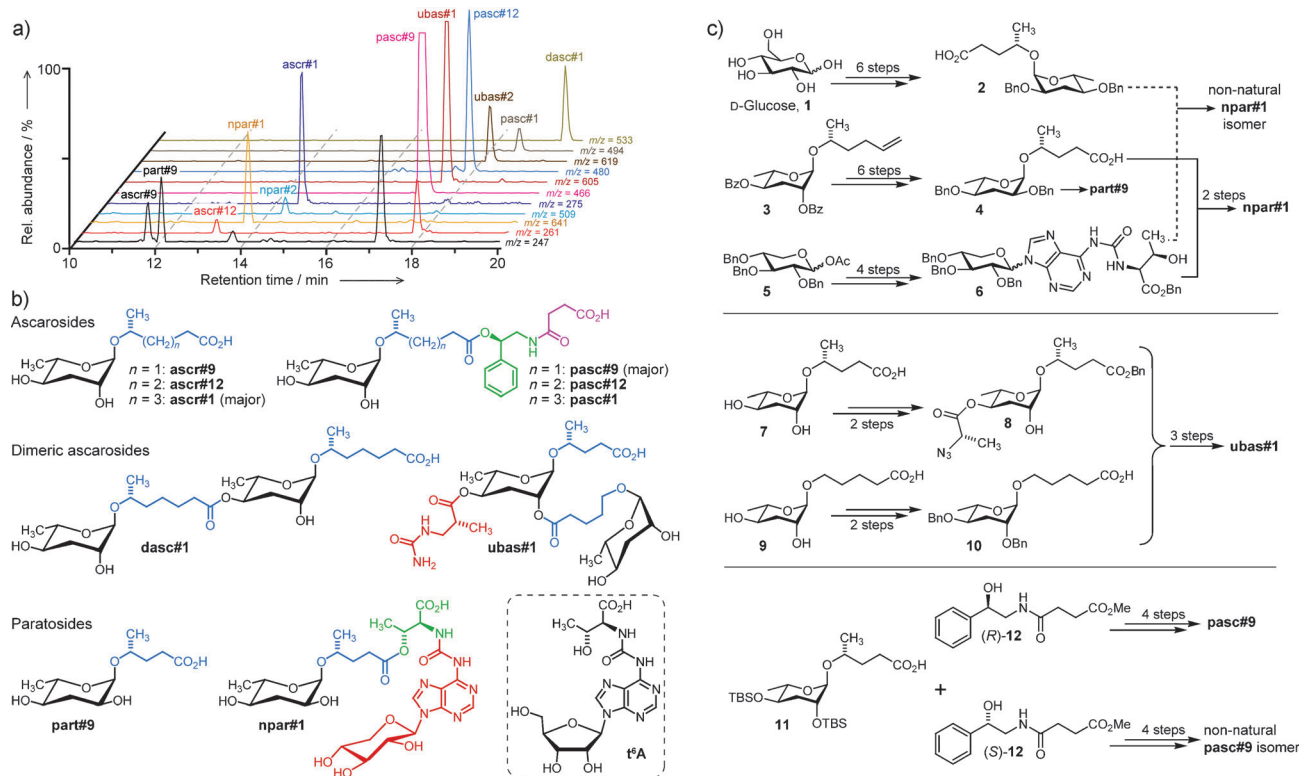


Figure 2. a) High-resolution HPLC-MS analysis reveals molecules whose molecular formulae provide additional constraints for structures proposed based on NMR-spectroscopic analysis of the *P. pacificus* exometabolome. b) Major components of the *P. pacificus* exometabolome derived from assembly of building blocks from fatty acid (blue), carbohydrate (black), amino acid (green), and nucleoside (red) metabolism, as well as TCA cycle-derived succinate (magenta). Also shown is the related tRNA nucleoside, *N*⁶-threonylcarbamoyladenine (t⁶A). c) Abbreviated syntheses for metabolites identified from *P. pacificus* and several non-natural stereoisomers (see Supporting Information).

thymine catabolism.^[12] Finally, we synthesized samples of the paratoside part#9 and the unusual xyloadenosine derivative, npar#1. By comparing the HPLC-retention times of natural part#9 and several synthetic part#9 diastereomers, we found that natural part#9 must be either D-paratosyl-4S-hydroxypentanoic acid or L-paratosyl-4R-hydroxypentanoic acid (Figure S6). We initially assumed that part#9 and npar#1 contained D-paratose, which had previously been described from bacteria whereas L-paratose had not been found in nature. Furthermore, D-paratose is a putative intermediate in the biosynthesis of L-ascarylose,^[11] on which all ascariosides in nematodes are based.^[4e,10,13] However, the HPLC retention time and NMR spectra of a synthetic npar#1 diastereomer including D-paratosyl-4S-hydroxypentanoic acid did not match the data obtained for natural npar#1 (Figure S7a). Therefore, we concluded that npar#1 must be based on L-paratosyl-4R-hydroxypentanoic acid, which was confirmed by synthesizing this diastereomer and comparing its spectroscopic data with those of natural npar#1 (Figure S7b,c). Using chiral derivatization agents (Mosher's acid chlorides),^[14] we further showed that part#9 is also based on L-paratose (Figure S8). The sugar L-paratose has not previously been found in nature; however, its occurrence in nematodes might result from epimerization of L-ascarylose at position 2.

To exclude the possibility that the identified compounds are bacterial metabolites, we additionally analyzed the metabolome of the *E. coli* OP50 bacteria used as food for *P. pacificus*. None of the identified compounds were found to be present in the bacterial metabolome (Figure S9). Furthermore, we showed that all identified compounds are also produced in *P. pacificus* cultures fed with *Pseudomonas sp.* instead of *E. coli* as well as in axenic (bacteria-free) cultures^[4d,15] (Figure S10).

Next we asked whether assembly of the identified small molecules from sugar, amino acid, lipid, and nucleoside-derived building blocks is selective. To address this, we carefully re-analyzed the entire *P. pacificus* exometabolome by high-resolution HPLC-MS/MS, quantified the identified compounds using synthetic standards (Table S1), and screened for homologues or alternative combinations of the primary metabolism-derived building blocks in our structures. We found that pasc#9 is accompanied by trace amounts of two homologues including six- and seven-carbon side chains, which were also detected by NMR spectroscopy (Figure S11). In addition, we detected a small amount of a homologue of ubas#1 as well as a derivative of npar#1 whose MS data indicated loss of the xylose (ubas#2 and npar#2 in Figure 2a, Table S1). Importantly, we did not observe any non-specific or seemingly random combinations of building blocks that would suggest a non-enzymatic genesis of the identified compounds.

We then tested synthetic samples of the identified compounds for their activity in the *P. pacificus* dauer- and mouth-form-dimorphism assays. As expected from previous studies that showed that *C. elegans* exometabolome samples are not active in the *P. pacificus* mouth-form dimorphism and dauer assays,^[16] we found that ascr#1, a compound abundantly excreted by *C. elegans*,^[4e,17] has no dauer-inducing activity in *P. pacificus*, even at concentrations higher than what is

physiologically observed (20 μM , Figure S12). In contrast, physiological concentrations of the nucleoside derivative npar#1 strongly induced dauer formation and appear to account for most of the reported dauer-inducing activity in the non-fractionated exometabolome (Figure 3a,c).^[9] Additionally, we observed weaker dauer induction with part#9 than

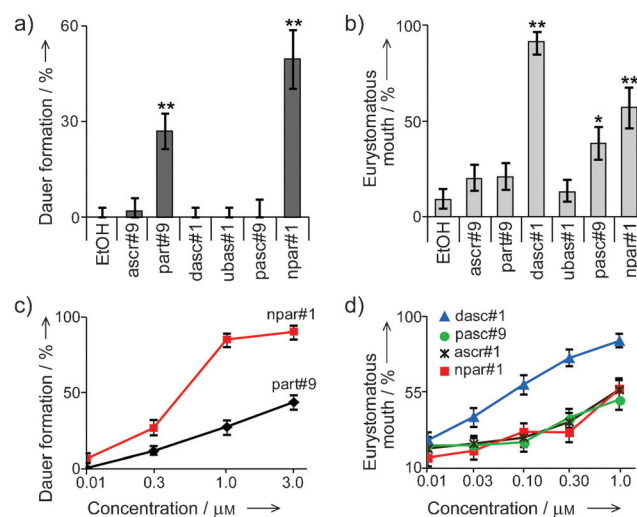


Figure 3. Regulation of mouth dimorphism and dauer induction by synthetic samples of identified *P. pacificus* metabolites. All experiments were performed in triplicate for each treatment. a,b) Compounds were assayed at 1 μM concentration (* = $p < 0.01$, ** = $p < 0.001$). c,d) Compounds with significant activity at 1 μM ($p < 0.01$) were subsequently tested at a range of concentrations.

with npar#1, whereas all other compounds tested were inactive in this assay. Testing our synthetic compounds in the mouth dimorphism assay, we found that the dimeric compound dasc#1, which was inactive in the dauer-formation assay, strongly induces the eury stomatous mouth form (Figure 3b,d). In addition, weaker induction of the eury stomatous mouth form was observed for high concentrations of pasc#9, ascr#1, and npar#1, whereas ascr#9 and part#9 as well as the dimeric ubas#1 were inactive at physiological concentrations in the wild-type strain tested (Figure 3b, Table S1).

These results show that adult phenotypic plasticity and larval development in *P. pacificus* are controlled by distinct yet partially overlapping sets of signaling molecules. Whereas mouth-form dimorphism is primarily regulated by dasc#1, the product of highly specific ascarioside dimerization, dauer formation is controlled by a molecule combining a paratoside with an unusual nucleoside. Previous work showed that the signaling molecules controlling phenotypic plasticity in *P. pacificus* act upstream of evolutionarily conserved transcription factors, including DAF-16/FOXO and the nuclear hormone receptor DAF-12 (Figure 4),^[18] whereby *daf-12* is required for both dauer induction and mouth-form dimorphism, whereas *daf-16*, is required for dauer induction but dispensable for regulation of mouth-form dimorphism.^[18a] Therefore, the different subsets of small molecules regulating dauer formation and mouth-form dimorphism appear to target different downstream effectors. Based on the recent

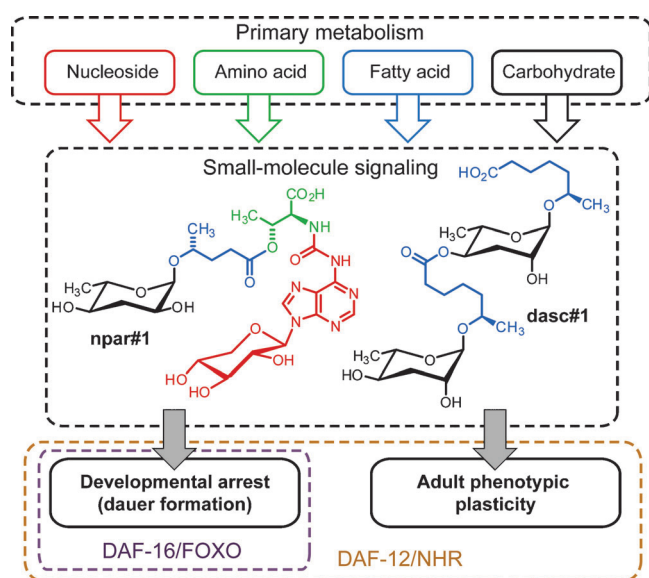


Figure 4. Small-molecule signaling in nematodes occupies a central position connecting primary metabolism to evolutionarily conserved transcription factors, including DAF-16/FOXO and the nuclear hormone receptor (NHR) DAF-12, a vitamin D and liver X receptor homologue.

identification of several G-protein coupled receptors (GPCRs) of the *C. elegans* dauer pheromone,^[19] it is probable that GPCRs expressed in specific chemosensory neurons connect npar#1 and dasc#1 with their respective downstream pathways in *P. pacificus*. Our results further demonstrate species-specific co-option of small molecule biosynthetic pathways for regulating different aspects of development, as ascr#1 contributes to dauer in *C. elegans*,^[17] whereas its dimer dasc#1 regulates adult morphology in *P. pacificus*. Similar to the multifunctional signaling properties of ascarosides in *C. elegans*,^[4] it is possible that dasc#1, npar#1, and the other identified compounds serve additional signaling functions in *P. pacificus*, for example, in mediating social behaviors and sex-specific attraction.

The identified small-molecules appear to integrate specific inputs from the major primary metabolic pathways: fatty acid, carbohydrate, amino acid, and nucleoside metabolism (Figure 4). Specific assembly of building blocks from these pathways using ester and amide linkages generates the modular molecules identified, which are further distinguished from previously known animal metabolites by the inclusion of L-paratose and xylopyranose-based adenosine. This unprecedented nucleoside is likely derived from metabolism of canonical (ribo)threonylcarbamoyl adenosine (t^6A , Figure 2b), a highly conserved nucleoside found directly adjacent to the anticodon triplet of a subset of tRNAs.^[20] t^6A plays an important role in maintaining translational fidelity; however, it usually accounts for only a very small fraction of tRNAs, and the production of large quantities of xylopyranose derivative in *P. pacificus* is surprising.

Our results provide direct evidence for small-molecule control of adult phenotypic plasticity in a metazoan that relies on conserved endocrine signaling pathways. Whether the biosynthesis of the identified signaling molecules involves

conserved pathways or depends on dedicated enzymes specific to *P. pacificus* (and perhaps other nematodes) is not known. However, the finding that the *P. pacificus* compounds are derived from assembly of modified primary metabolites suggests that their biosynthesis is largely based on conserved biochemical pathways (Figure 4). Notably, the *P. pacificus* genome contains more than 25000 predicted genes with many specific gene duplication events among genes encoding primary metabolic enzymes.^[21] Supporting the involvement of primary metabolism in the biosynthesis of nematode signaling molecules, recent investigations of ascaroside biogenesis in *C. elegans* showed that the lipid-like ascaroside side chains are derived from conserved peroxisomal- β -oxidation.^[4e,22] Known signaling molecules and co-factors in higher animals, for example, S-adenosyl methionine or phosphatidylinositols, often rely on the combination of building blocks derived from one or two different primary metabolic pathways. Our results demonstrate that metazoans may extend such strategies to produce signaling molecules of much greater structural complexity, suggesting that detailed spectroscopic re-analysis of metabolomes from higher animals, including mammals, may also reveal novel types of modular small-molecule signals.

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